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# Investigations on the occurrence of *Plasmodium knowlesi* in travellers returning from the endemic areas of simian malaria

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## ABSTRACT

*Malaria remains an important public health issue all over the world. Among 5* Plasmodium *species invasive to humans,* Plasmodium knowlesi *has been identified most recently. It is sometimes difficult to differentiate this species from* P. malariae *with the use of microscopic examination. However,* P. knowlesi *infection may be associated with rapidly increasing parasitaemia and severe clinical course with the risk of death. Samples from Polish travellers returning from areas where simian malaria is endemic were examined with the use of polymerase chain reaction (PCR). The small subunit of ribosomal RNA (SSU rRNA) genes was subjected to analysis using nested PCR reaction.*

*No positive results of* P. knowlesi *were obtained. Due to morphological similarities to* P. malariae*, potentially severe clinical course of infection and* P. knowlesi *endemic regions being a common tourist destination, diagnostic and clinical vigilance is necessary, including molecular methods use for precise parasite identification.*

**Key words: simian malaria, *Plasmodium knowlesi*, travel medicine, tropical diseases, PCR**

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## INTRODUCTION

Malaria still remains a serious public health challenge in the tropics. Despite governmental and non-governmental organisations efforts to decrease the number of malaria cases in the world, still almost 200 million symptomatic infections are diagnosed yearly with estimated 584,000 deaths (as at 2014) [1]. There are over than 200 species of *Plasmodium* that infect different hosts [2]. Only 4 species, namely *Plasmodium falciparum, P. vivax, P. malariae*, and *P. ovale* are well-known infectious agents that cause this disease in humans. The fifth species — *P. knowlesi* — a simian malaria parasite species commonly found in long-tailed and pigtailed macaques (*Macaca fascicularis* and *Macaca nemestrina*, respectively) is the only malaria parasite affecting primates with a 24-h erythrocytic cycle [3, 4]. *P. knowlesi* is transmitted by *Anopheles leucosphyrus* group [5]. Simian malaria parasites were first reported in Malayan monkeys by Daniels in 1908 [6]. In 1965 the first natural infection in human was reported in a man from the United States who was infected in the jungles of Pahang, Malaysia [7]. In 2004, Singh et al. [8] reported the presence of a large number of *P. knowlesi* cases in Sarawak, a state in Malaysian Borneo. Human infections with *P. knowlesi,* a simian malaria parasite, are more common than previously thought [9]. *P. knowlesi* malaria appears to be the most common malaria species in Malaysian Borneo and is also widely distributed on the Malaysian mainland. Moreover, locally transmitted cases of simian malaria in humans have been reported in Thailand, the Philippines, Vietnam, Singapore, Myanmar, Indonesian Borneo, Brunei, Cambodia and

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Andaman and Nicobar Islands of India. Two cases have been reported from non-endemic countries in Asia (Japan and Taiwan) in people with a history of travel to Malaysia and the Philippines. Twelve cases were imported to their home countries by travellers from other continents: 2 from the United States, 2 from the Netherlands, 2 from Germany, and 1 each from Spain, France, Sweden, Finland, Australia, and New Zealand. In most cases, the infection was associated with a trip to or near forested areas [3, 10].

The "gold standard" for the identification of *Plasmodium* species is microscopic examination because it is relatively rapid and inexpensive. However, because of similarity of *P. knowlesi* and *P. falciparum* in the early trophozoite stage and with *P. malariae* in the late and mature trophozoites, gametocytes and schizonts, large numbers of false negative results is observed [9, 10]. The high rate of misdiagnosis of *P. knowlesi* infection as *P. falciparum* and *P. malariae* using light microscopy in non-endemic countries should lead to molecular diagnostic techniques [10]. Rapid diagnostic tests are becoming an increasingly popular method for diagnosing malaria, particularly by medical laboratory technologists in non-malaria endemic countries, who are not experienced in identifying malaria parasites by microscopy [11, 12]. It is possible that the low parasitaemia may have been a contributing factor to the inability of the rapid diagnostic test to detect simian malaria, because these tests are not as sensitive as light microscopy [13].

The aim of the present study was retrospective identification of *Plasmodium knowlesi* species using polymerase chain reaction (PCR) in diagnostic specimens, in Polish travellers with malaria infection, returned from areas where simian malaria is endemic or whose history of travelling was not known.

## MATERIALS AND METHODS

A retrospective study was performed on blood samples from patients treated at the University Centre for Maritime and Tropical Medicine in Gdynia during the period 1995–2013. Routine laboratory diagnosis (thick and thin Giemsa stained blood smears) was performed at the Department of Tropical Parasitology, Institute of Maritime and Tropical Medicine, Medical University of Gdansk, Poland.

### DNA EXTRACTION

DNA extraction from collected blood samples was performed using a Blood Mini Kit (A&A Biotechnology, Poland), according to the manufacturer's instructions. The PCR templates were stored at –20°C.

### SPECIFIC DETECTION OF *PLASMODIUM* SPECIES BY NESTED PCR

The small subunit of ribosomal RNA (SSU rRNA) genes was subjected to analysis using nested PCR reaction. The first step was performed using the *Plasmodium* genus specific primers rPLU1 and RPLU5 [14]. The PCR amplification was performed in a 50 µL reaction mixture, which contained 5 µL 10 × Reaction Buffer for RUN (A&A Biotechnology, Poland), 0.25 mM of each dNTP (Fermentas, Lithuania), 0.2 µM of each primer (Metabion, Germany), 1 U of RUN polymerase (A&A Biotechnology, Poland), and 1 µL of DNA template. The amplification conditions were as follows: 95°C for 5 min, 25 cycles at 94°C for 1 min, 58°C for 2 min, 72°C for 2 min, followed by a final extension for 5 min at 72°C. The second step of PCR reactions was performed using primers Pmk8 and Pmkr9 (specific to *P. knowlesi*), FAL1, FAL2 (specific to *P. falciparum*), VIV1, VIV2 (specific to *P. vivax*), MAL1, MAL2 specific to *P. malariae*), OVA1, OVA2 (specific to *P. ovale*) [4, 14] in a 25 µL reaction volume containing 2,5 µL 10 × Reaction Buffer for RUN (A&A Biotechnology, Poland), 0.25 mM of each dNTP (Fermentas, Lithuania), 0.2 µM of each primer (Metabion, Germany), 1 U of RUN polymerase (A&A Biotechnology, Poland), and 1 µL of DNA template. The PCR conditions were the same as described in the first round except that the number of cycles was 35. All PCR experiment were performed with the use of positive control (DNA isolated from infected patients) in case of *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* control and a plasmid containing synthetic fragment of *P. knowlesi* sequence prepared by laboratory (DNA-Gdansk, Poland) and double distilled water instead of a DNA template as a negative control. The PCR amplifications were run in a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, USA). The PCR products were analysed using a Gel Doc-It Imaging System (UVP, USA) after electrophoresis on a 2% gel agarose, which was stained with Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany).

## RESULTS

A retrospective study was performed on blood samples from 9 patients. No cases of *P. knowlesi* were diagnosed in the samples included into the study, while *P. vivax*, *P. malariae* and *P. falciparum* were detected (Table 1).

## DISCUSSION

International travel, both for recreational and business purposes, has been rapidly increasing for last few decades. In the 1960s only 75 million people travelled abroad every year, while in 2000 this number reached 175 million [15]. Destinations include all parts of the world, including tropical regions where malaria and other tropical diseases are endemic. A rise in the number of trips to tropical countries is associated with increased risk of importing rare parasitic diseases to Europe.

There is a growing interest in travelling among Polish citizens, including journeys to exotic off the beaten track or dangerous destinations. Moreover, there is a significant

**Table 1.** Malaria infection in Polish travellers returning from endemic areas of simian malaria diagnosed in the Department of Tropical Parasitology Institute of Maritime and Tropical Medicine, Medical University of Gdansk, Poland

| Patient | Year | Blood smear | Polymerase chain reaction | Destination |
|---|---|---|---|---|
| 1 | 1995 | *P. vivax,* single trophozoites | *P. vivax* | Oceania + ? |
| 2 | 1996 | *P. vivax,* early trophozoites (rings), mature trophozoites, schizonts | *P. vivax* | Papua New Guinea + ? |
| 3 | 1996 | *Plasmodium* sp., *P. vivax*? single mature trophozoites | *P. vivax* | Southeast Asia |
| 4 | 1996 | *P. vivax*, rings, mature trophozoites, gametocytes | *P. vivax* | Southeast Asia |
| 5 | 1999 | Negative | *P. malariae* | ? |
| 6 | 2001 | *P. vivax*, gametocytes | *P. vivax* | Indonesia |
| 7 | 2001 | *P. falciparum*, rings | *P. falciparum* | ? |
| 8 | 2002 | *P. vivax*, rings, mature trophozoites | *P. vivax* | Southeast Asia |
| 9 | 2013 | *P. vivax*, gametocytes | *P. vivax* | ? |

number of missionaries who travel to several geographic locations worldwide and also contract employees working overseas. In 2012, 25,032,708 tourist arrivals were registered in Malaysia. Most of them were from Asia, especially from Singapore, Thailand, Indonesia, Brunei, and China. In the same year, Tourism Malaysia reported an increase in the number of travellers: 1,002,067 from Europe (also from Poland), 327,065 from the United States and Canada, and 573,674 from New Zealand and Australia [15].

In Poland, approx. 20 to 50 imported cases of malaria are diagnosed yearly [16–18], and the number of reported malaria cases remains low compared to countries of Western Europe. 30% of them, that is about 10 persons per year, are admitted to the University Centre for Maritime and Tropical Medicine in Gdynia [own study, unpublished data]. Among all malaria patients diagnosed and treated in Gdynia in 1984–1994, 16.84% were travellers returned from Asia [17]. In the Department and Clinic of Tropical and Parasitic Diseases, Medical University of Poznan in the years 2001–2013 this number reached 7.1%, while in the Department of Infectious Diseases and Hepatology, Pomeranian Medical University in Szczecin in the years 2010 and 2011 it was 27.3% [19]. It can be assumed that some of these patients might have returned from region endemic for simian malaria and thus the prevalence of *P. knowlesi* in humans may be underestimated [9].

Malaria mortality rates vary between different countries. In the United Kingdom case fatality rate (in *P. falciparum* infections) is 1% and this value is similar in most developed countries [20]. In Poland case fatality rate in the year 2004 was 3.4%, while in 2008 it was 4.3% [16]. These differences can be explained by lack of clinical experience in malaria treatment (due to a low number of imported malaria cases in Poland) as well as non-compliance with antimalarial prophylaxis among Polish travellers.

Treatment in cases of *Plasmodium* infection mainly depends on the patient's condition, the species of *Plasmodium* and the potential drug resistance. *P. falciparum* is considered the most dangerous species, requiring intensive therapeutic measures from the treating physician. There are also reports on severe course of *P. vivax* infections, sometimes inappropriately called "benign" [21], such cases have also been observed in the Clinic of Tropical and Parasitic Diseases Institute of Maritime and Tropical Medicine, Medical University of Gdansk, Poland. *Plasmodium ovale* and *P. malariae* infections usually have an uncomplicated course and the patient's condition is usually stable.

The mature trophozoites, schizonts and mature gametocytes of the described parasite *P. knowlesi* are practically indistinguishable from *P. malariae* in microscopic examination, therefore, misidentification of the parasite may give the treating physician a false sense of security, whereas *P. knowlesi* infection has a course similar to *falciparum* malaria and may result in sudden deterioration of patient's condition and multi-organ failure [22]. Numerous reports on the difficulties in microscopic distinguishing of *P. knowlesi* from *P. falciparum* or *P. malariae* and on the consequences of misidentification exist in the literature [12, 23–25]. It is believed that microscopic examination without molecular confirmation has limited reliability e.g. in distinguishing between *P. knowlesi/vivax/falciparum* which leads to inappropriate treatment choices such as administering chloroquine in *falciparum* malaria or not prescribing primaquine in *P. vivax* infection. In the areas endemic for *P. knowlesi*, treatment unification for all malaria patients regardless of the parasite's species has been advocated, along with the use of molecular techniques of species identification [26]. In non-endemic areas, patients returning from South-East Asia have been reported to be infected with *P. knowlesi*. Compli-

cations typical for *falciparum* malaria, such as renal insufficiency, may occur even at low parasitaemia. In a German patient, renal failure was observed at parasitaemia of 0.2%. A 55-year-old woman with fever, nausea and vomiting 10 days after a holiday in Thailand, presented acute kidney injury and elevated procalcitonine level. In stained thin and thick blood films, plasmodia resembling *P. malariae* were present with 0.2% trophozoite parasitaemia and numerous gametocytes. With a diagnosis of malaria and acute renal failure, the patient was referred to the Tropical and Infectious Diseases Service. Monoinfection of *P. knowlesi* was confirmed by 3 techniques: PCR protocols based on 18sRNA, mitochondrial DNA, the linker region of dihydrofolate reductase and thymidylate synthase. Intravenous treatment with artesunate (2.4 mg/kg) was started and switched to oral treatment with artemether/lumefantrine. The patient recovered promptly with no sign of renal insufficiency [25].

No simian malaria cases were detected in our study. The majority of the patients in Poland were infected with the most severe *P. falciparum* species [17–19]. In patients with suspected or confirmed malaria, it is important to collect their epidemiological history for further diagnostics and parasite species identification in order to administer adequate treatment. It is crucial to identify *P. knowlesi* infections, especially in the late stage when the parasites resemble *P. malariae*, because *P. knowlesi* infections can sometimes be associated with complications and may be fatal.

In Poland malaria is diagnosed most often with the use of microscopic examinations of blood films and rapid immunochromatographic tests. In low parasitaemia cases microscopic examinations may not be enough to identify the parasite species in mixed infections [27] and due to morphological similarities of *P. knowlesi* to *P. falciparum* and *P. malariae* adequate diagnosis is crucial for treatment selection and patient outcome. It has been suggested, that *Plasmodium* species identification is important not only for treatment modification in case of two species capable to develop hypnozoites (*P. vivax, P. ovale*), but also because of potentially severe clinical course of *P. knowlesi* malaria. Asexual stages of *P. knowlesi* are difficult to differentiate from *P. malariae*. The latter species however is not associated with high parasitaemia and results in uncomplicated malaria only. Thus in cases of malaria initially suspected as *P. malariae,* and with the epidemiological history pointing to the risk for simian malaria, potentially severe clinical course of infection should be considered and appropriate measures introduced, including patient monitoring and implementing proper medical care. For detailed assessment of aetiological agent, parasite species identification is necessary and for this purpose molecular techniques are very useful and usually available in research units [28].

## CONCLUSIONS

Physicians should be aware that simian malaria infection is an important differential diagnosis in febrile travellers with a recent travel history to Southeast Asia, who tested negative with rapid diagnostic tests or blood smears.

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